Harmonization of Light Scatter and Fluorescence Flow Cytometry Profiles Obtained After Staining Peripheral Blood Leucocytes for Cell Surface-Only Versus Intracellular Antigens with the Fix & Perm™ Reagent

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Staining for intracellular markers with the Fix & Perm™ reagent is associated with variations in the scatter properties of leucocytes, limiting automated analysis of flow cytometry (FCM) data. Here, we investigated those variables significantly contributing to changes in the light scatter, autofluorescence, and bcl2 staining characteristics of peripheral blood (PB) leucocytes, after fixation with Fix & Perm™. Our major aim was to evaluate a new mathematical approach for automated harmonization of FCM data from datafiles corresponding to aliquots of a sample treated with cell-surface-only versus Fix & Perm intracellular staining techniques. Overall, neither the anticoagulant used nor sample storage for <24 h showed significant impact on the light scatter and fluorescence properties of PB leucocytes; similarly, the duration of the fixation period (once >15 min were used) had a minimum impact on the FCM properties of PB leucocytes. Conversely, changes in cell/protein concentrations and the fixative/sample (vol/vol) ratio had a clear impact on the light scatter features of some populations of leucocytes. Accordingly, lower cell/protein concentrations were associated with lower scatter values, particularly for the neutrophils. Such changes could be partially corrected through the use of higher fixative to sample vol-

This article is dedicated to the achievements of Prof. Walter Knapp, founding member of the EGIL group, who made it possible to routinely analyze intracellular markers by flow cytometry.

The first two authors have contributed equally to this work.

Conflict of interest: Cytognos S.L. is part of the UE-supported EuroFlow Research Consortium, and has implemented some of the algorithms described in this study, in its proprietary software INFINICYT; Cytognos S.L. has a contract license of several patents owned by the University of Salamanca, of which A. Orfao, C.E. Pedreira, and E.S. Costa are inventors. Other authors declare no competing financial interests.

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ume ratios. Despite the variable changes detected between aliquots of the same sample treated with cell surface-only versus intracellular staining procedures, the new mathematical approach here proposed and evaluated for automated harmonization of common parameters in both datafiles, could correct the FCM profiles of leucocytes derived from cells undergoing conventional fixation/permeabilization procedures, and made them indistinguishable from those corresponding to aliquots of the same sample treated with cell-surface-only staining techniques.

Key terms: light scatter; intracellular proteins; immunofluorescence; flow cytometry; cell fixation; automation; eigenvectors

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INTRODUCTION

At present, flow cytometry (FCM) is the preferred method for the immunophenotypic analysis of neoplastic hematopoietic cells (1–3). For many years, immunophenotypic analysis of leukemic cells by FCM was almost restricted to the evaluation of cell surface antigens (4). This was mainly due to the lack of reproducible intracellular staining procedures that could be universally applied for the detection of the most relevant nuclear and cytoplasmatic proteins (5). During the 90s, several new intracellular staining procedures based on sequential fixation and permeabilization of nucleated cells, have become available (6–8). Once compared, these intracellular staining approaches show significantly different sensitivities for the detection of intracellular proteins, due to a variable effect on increasing cellular background autofluorescence and decreasing specific antigen-associated fluorescence of different cell populations coexisting in blood-containing samples (9,10). In addition, the use of such intracellular staining techniques is also associated with variable changes in the light scatter characteristics of both normal and malignant hematopoietic cells from both peripheral blood (PB) and bone marrow (BM) (9,10).

Information about the light scatter characteristics of cells present in a sample, in combination with their immunophenotypic properties, is essential for the classification of cellular events measured by multiparameter FCM into different cell populations. Accordingly, changes in light scatter and fluorescence profiles induced by cell fixation/permeabilization procedures required for intracellular stainings, frequently display a negative impact on the identification of the different cell populations present in a sample (11,12); in addition, since significant variation is observed when different sample preparation techniques are used to stain different aliquots of a sample for intracellular markers (9), it also increases the difficulty in comparing the flow cytometric characteristics of a cell population from different aliquots of the same sample, stained with conventional approaches for intracellular versus surface-antigen-only procedures. Because of this, the use of different regions for the identification of the same cell population is frequently required in surface-only versus intracellularly stained aliquots of the same sample (13). This becomes more relevant when either automated data analysis procedures are applied, or information is calculated for cellular events in one aliquot of a sample, based on the phenotypic characteristics identified for the same cell population in another aliquot of the same sample, as described by Pedreira et al. (14) using the nearest-neighbor principle (15,16).

In this study, we evaluate the impact of different cell fixation/permeabilization associated variables on the light scatter and fluorescence characteristics of PB nucleated cells. Our interest did not focus on repeating and extending on the effect of different combinations of cell fixation/permeabilization reagents on the expression of multiple antigens, but on evaluating a new strategy for automated harmonization of FCM data from different aliquots of a sample measured after using different sample preparation techniques for cell-surface-only versus intracellular staining of bcl2 with the Fix & Perm™ reagent.

MATERIALS AND METHODS

Samples and Variables Under Study

PB samples from five healthy adult volunteers (four men and one woman; mean age of 34 ± 10 years) were obtained after informed consent. For each case, both EDTA- and heparin-anticoagulated samples were obtained between 9:00 and 10:00 am.

For each sample, the impact of five different variables (or combinations of variables) on the light scatter, autofluorescence and bcl2 staining properties of the major populations of PB lymphocytes, monocytes, neutrophils, and eosinophils were evaluated, after fixation/permeabilization with the Fix & Perm™ reagent kit (Invitrogen, Carlsberg, CA); cell-surface-only staining of an aliquot of the same samples, was taken as reference. The five variables and combinations of variables under evaluation

included: (1) type of anticoagulant: EDTA versus heparin-anticoagulated and freshly obtained (<2 h) PB samples (time 0) were prepared in parallel; (2) time from sample collection (EDTA-anticoagulated samples): preparation of the same sample at time 0 versus after storage for 24 h at room temperature; (3) varying periods of incubation of recently collected EDTA-anticoagulated samples with the fixative: 10, 15, 20, and 30 min; (4) fixative to sample volume ratio: increasing volumes (10, 25, 50, 75, 100, 150, 200, and 300 μL) of fresh (time 0) EDTA-anticoagulated PB samples to which the same amount (100 μL) of fixative was added, were prepared and measured in parallel; and (5) varying cell/protein concentration in lower fixative to sample volume ratios: staining progressively higher volumes of PBS-diluted PB (10, 25, 50, 75, 100, 150, 200, and 300 μL) containing identical numbers of cells, as prepared by diluting 10 μL of EDTA-anticoagulated PB collected at time 0 with increasing volumes (0, 15, 40, 65, 90, 140, 190, and 290 μL, respectively) of filtered phosphate buffered saline (PBS; pH = 7.6) to which 100 μL of fixative was added.

Immunophenotypic Studies

For all different measurements performed, samples were processed using the Fix & Perm™ intracellular detection kit, strictly following the recommendations of the manufacturer, except when different sample-to-fixative volume ratios were evaluated, as described above. Briefly, 100 μL of the fixative solution (solution A) was added to 100 μL of whole PB (containing around 0.5 to 1 × 10^6 white blood cells) and incubated for 15 min at room temperature (RT); after a washing step (5 min at 540 g), both 100 μL of the permeabilizing solution (solution B) and 5 μL of fluorescein isothiocyanate (FITC)-conjugated anti-bcl2 monoclonal antibody (clone 124, DAKO, Glostrup, Denmark) were added to the cell pellet and another incubation for 15 min (RT) in the darkness, was performed. In all cases, a replicate of each sample aliquot which did not contain the anti-bcl2 monoclonal antibody reagent, was prepared in parallel in an otherwise identical way, to evaluate baseline green autofluorescence levels of the different populations of PB cells analyzed. In addition, an ammonium chloride lysed (2 mL) and washed (5 min at 540 g in 2 mL of PBS) unstained aliquot of each PB sample (100 μL), was prepared in parallel.

Data acquisition was performed in a FACScalibur flow cytometer—Becton/Dickinson Biosciences (BDB), San Jose, CA—using the CellQUEST software program (BDB); the Infinicyt (Cytognos SL, Salamanca, Spain) and Paint-A-Gate PRO software (BDB) programs were used for data analysis. The major populations of PB leucocytes analyzed (eosinophils, neutrophils, monocytes, and lymphocytes) were identified on the basis of their different light scatter—forward light scatter (FSC) and exponentially transformed sideward light scatter (SSC) (17) and autofluorescence characteristics, as described in Figure 1; although FL2-associated orange fluorescence was used here for the gating strategy, it is not essential and could be replaced by other specific markers. For each population of PB leucocytes, results were reported as amount of FSC and SSC, expressed as their mean channel number (arbitrary linear units scaled from 0 to 1023) and mean amount of FL1-associated green fluorescence (autofluorescence and bcl2-FITC-associated green fluorescence) expressed as mean fluorescence intensity (MFI; arbitrary relative linear units scaled from 0 to 10,000).

Automated Correction of Light Scatter and Fluorescence-Associated Parameters

Automated correction of FSC, SSC, and fluorescence-associated parameters was performed for specific cell populations after they were manually gated by an expert operator using dedicated software (Paint-A-Gate and Infinicyt). For this purpose, the same cell populations were gated separately in data-files corresponding to aliquots of the same sample treated in parallel for cell surface staining-only and intracellular procedures. For each group of paired data files two matrices (X and W) were built where columns contained information about different parameters, whereas individual events from the gated cell population were represented in horizontal lines. Matrix X contained data for cell surface staining-only procedures, whereas matrix W corresponded to paired data obtained after intracellular staining procedures. For the purpose of detailing the calculations, let us assume that these matrices store n and m observations, respectively. Then the two sets of events corresponding to the cell surface-only staining technique and to the intracellular staining approach were both translated to a reference point (namely, the origin). For this purpose, we subtracted the mean value obtained for each parameter, for the population of events in which each individual event was included, from the value obtained for each individual event for that specific variable. Accordingly, we defined two new matrices X^0 and W^0 with lines:

$$x_j^0 = x_j - \frac{1}{n}\sum_{i=1}^{n} x_i \qquad \text{and} \qquad w_r^0 = w_r - \frac{1}{m}\sum_{i=1}^{m} w_i.$$

Afterward, two sets of eigenvectors (18) A_X^0 and A_W^0 were calculated for the covariance matrices of the set of observations stored in the X^0 and W^0 matrices. These eigenvectors were derived using the respective eigenvalues in the decreasing sense. Assuming that the used parameters are linear independent (i.e., they cannot be written as a linear combination of two or more parameters), the vectors in A_X^0 and A_W^0 form two bases for the linear space embedding the corresponding observations. Accordingly, any combination of a vector and the corresponding observations in these spaces may be written as a linear combination of either A_X^0 or A_W^0. So, any observation in a base may be specified by its coordinates with respect to this base. It should be noted that, for an observation in a given space, different bases imply different coordinates. The procedure we describe and use in this study reproduces the set of observations from X^0, in

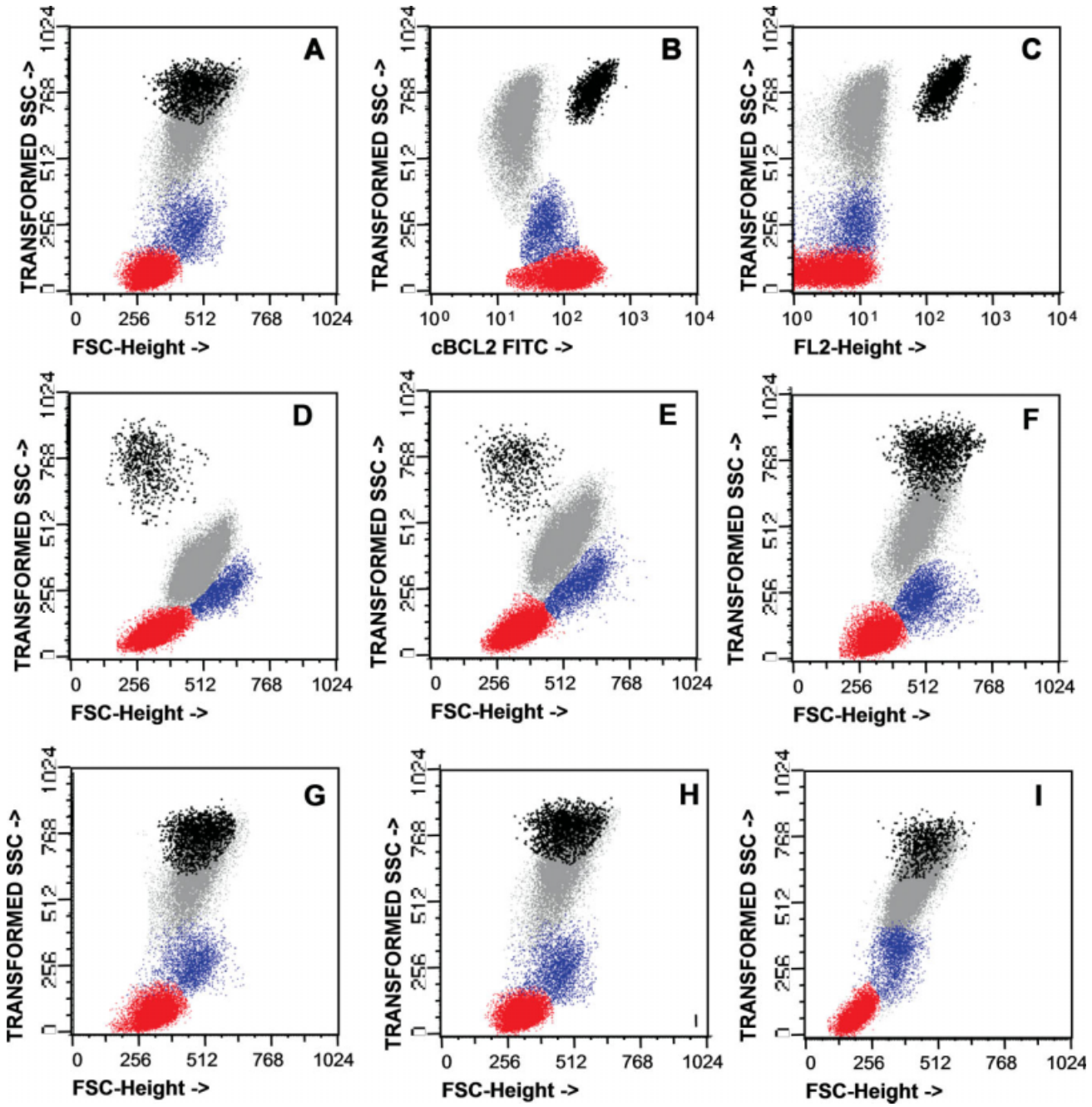

FIG. 1. Bivariate dot plot histograms illustrating the gating strategy used for the identification of peripheral blood neutrophils (gray dots), lymphocytes (red dots), monocytes (blue dots), and eosinophils (black dots). In Panels **A–C**, the gating strategy is illustrated: eosinophils were identified as displaying the highest SSC values with increased orange (FL2-height) autofluorescence; in turn, lymphocytes showed the lowest FSC and SSC and orange autofluorescence values with relatively high and heterogeneous bcl2 expression. Finally, the distinction between monocytes and neutrophils was based on the higher FSC and lower SSC of the former cell population. The visual effect of using progressively lower fixative/sample (vol/vol) ratios for fixation of increasingly high numbers of cells (0.53, 1.33, 2.65, 5.3, 15.9×10^5 leucocytes/tube) on the side scatter–SSC (arbitrary channel unit values), represented as exponentially transformed SSC (17) characteristics of peripheral blood neutrophils is illustrated in Panels **D–H** for a normal peripheral blood sample. In Panel **I**, the FSC versus SSC characteristics of fixed but nonpermeabilized leucocytes is displayed for visual comparison.

the A^0_W base. Therefore, for each observation in X^0 its coordinates in base A^0_X were identified and these same coordinates were used to represent each observation from the A^0_W base. As an example, if C^0_X is the coordinate of the observations stored in X^0, these observations may be written in base A^0_X as $X^0 = C^0_X\ (A^0_X)$; thus, the coordinates of X^0 in base A^0_X may be calculated as $C^0_X = X^0\ (A^0_X)^{-1}$. Now, the coordinates can be used to

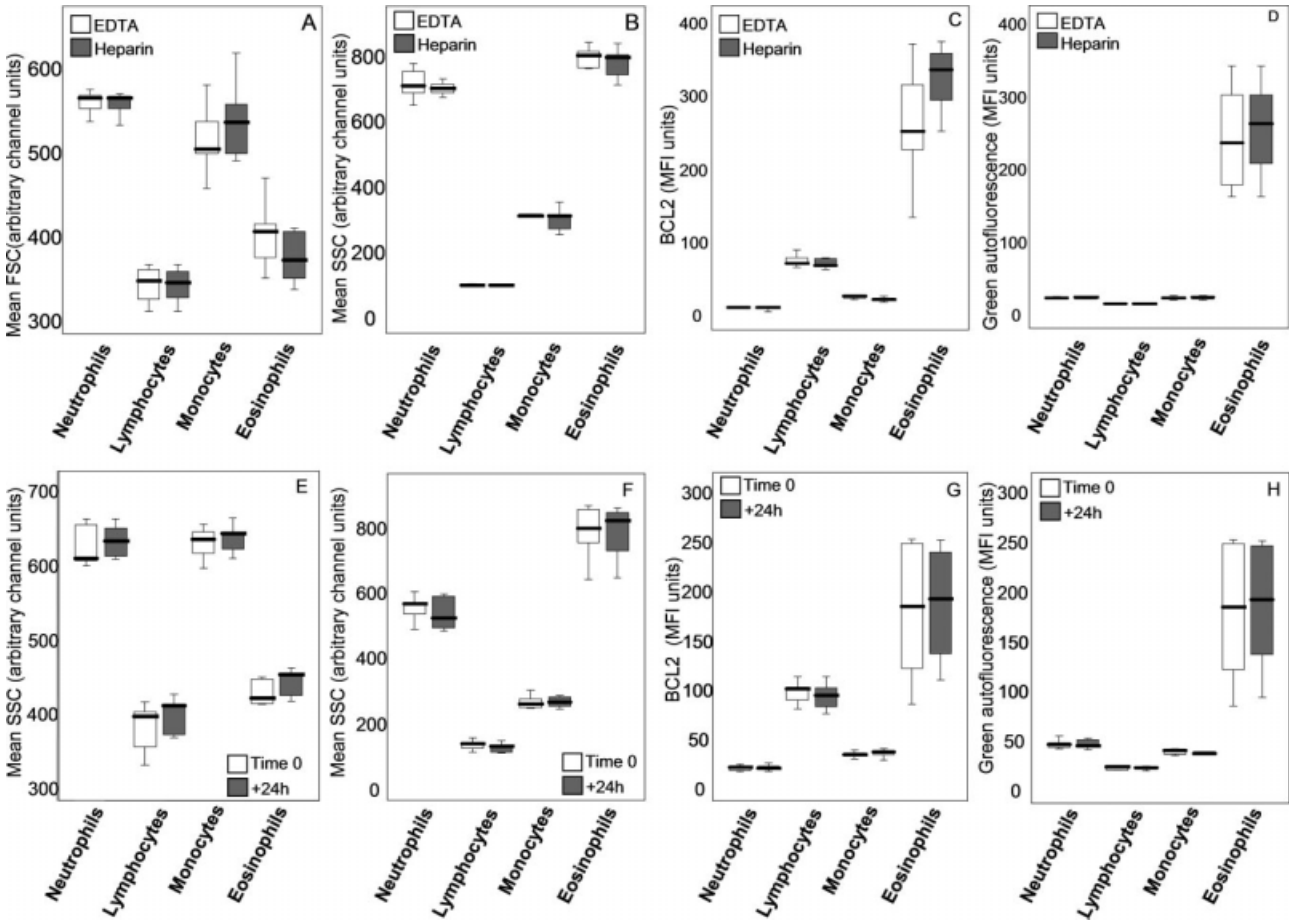

FIG. 2. Effect of the type of anticoagulant (EDTA versus heparin; Panels **A**–**D**) and time from collection of EDTA-anticoagulated peripheral blood (PB) samples (time 0 vs. +24 h; Panels **E**–**H**) on the light scatter characteristics (arbitrary linear mean channel values, scaled from 0 to 1023), the intensity of expression of bcl2 and the levels of FL1-associated green autofluorescence—arbitrary mean fluorescence intensity (MFI) values, scaled from 0 to 10,000—of different populations of fixed and permeabilized PB leucocytes. Notched boxes represent 25th and 75th percentile values; the line in the middle and vertical lines correspond to the median value and both the 10th and 90th percentiles, respectively. No statistically significant differences ($P > 0.05$) were noted for any of the comparisons performed.

represent observations in base A^0_W as $X^0_h = C^0_X(A^0_W)$. In this way, X^0 observations were translated to the W^0 space in such a way that their relative positions were maintained.

In the calculation process, it is assumed that the two matrices correspond to the same population after suffering different levels of distortion due to the use of different sample preparation procedures. Therefore, it might be assumed that for the two matrices, those eigenvectors associated with the largest eingenvalues must be close to each other. To avoid that corresponding eigenvectors in the two matrices have opposite senses (which may happen in the calculations), we limited the distance between the eigenvectors: if this limit was violated, then, one of the vectors was inverted by multiplying its value by -1. In practice, the two sets of eigenvectors form two different bases (19) that generate two coordinate systems, one for the "cell surface-only" and the other for the "intracellular" spaces. Events from the intracellular staining datafile were represented in the coordinate system generated by the cell-surface staining eigenvectors, to adjust the two cell populations into the same frame of reference. Finally, we ended-up adding back to each event the mean value obtained for the cell population represented by the cell surface technique to translate back the pair of cell populations corresponding to the "cell-surface-only" and the "intracellular" stained aliquots, to the original and corrected positions, respectively. At this point, it should be noted that one does not exactly return the events from the intracellular staining data file to their initial position, since they are now represented in the coordinate system generated by the cell-surface staining eigenvectors (18).

Statistical Methods

For all parameters studied, mean values ± one standard deviation, median, range and the 25th and 75th percentiles were calculated using the SPSS (V12) program (SPSS, Chicago, IL). The statistical significance of the differences observed among distinct groups of sample aliquots was calculated using the paired Wilcoxon's or Friedman nonparametric tests for two or more than two

variables, respectively. P values <0.05 were considered to be associated with statistical significance.

RESULTS

Impact of Sample Preparation Procedures on the FCM Features of Different Populations of PB Leucocytes

As shown in Figure 2 (Panels A–D), no statistically significant ($P > 0.05$) differences were found between EDTA- and heparin-anticoagulated samples, either in the light scatter characteristics or in the intensity of expression of bcl2-FITC and FL1-associated green autofluorescence, for any of the major PB leucocyte populations analyzed (neutrophils, lymphocytes, monocytes, and eosinophils). However, clearly higher numbers of events corresponding to debris and platelets were found in heparin- versus EDTA-anticoagulated samples (data not shown); this observation prompted us to use EDTA samples for further comparisons, which are described later.

In a similar way, storage of EDTA-anticoagulated samples for 24 h (RT) after they had been collected, did not induce significant ($P > 0.05$) changes in the FSC, SSC, bcl2-FITC, and green autofluorescence characteristics of PB neutrophils, lymphocytes, monocytes, and eosinophils when compared with fresh samples (time 0) (Fig. 2, Panels E–H).

Overall, the duration of the incubation period of the sample with the fixative (10 vs. 15 vs. 20 vs. 30 min) did not show a significant ($P > 0.05$) impact on the light scatter characteristics, green autofluorescence levels, and bcl2-FITC expression, for any of the different populations of PB leucocytes analyzed, except for slightly increased levels of FSC ($P = 0.02$) and SSC ($P = 0.03$) found for the monocytes and a higher mean lymphocyte SSC ($P = 0.03$), once incubation periods of 10 versus $\geq$15 min were used (Fig. 3).

Fixation of an increasingly higher number of cells (increasing cell/protein concentrations) at progressively lower fixative/sample (vol/vol) ratios, showed significantly lower FSC ($P = 0.01$) and higher SSC values for the neutrophils ($P = 0.0001$) and slightly decreased SSC for the monocytes ($P = 0.03$) (Figs. 1 and 4). In turn, the light scatter characteristics of the eosinophils and the lymphocytes were relatively stable and independent of the cell/protein concentration and fixative/sample volume ratios tested (Fig. 4). Interestingly, progressively greater cell/protein concentrations with lower fixative/sample volume ratios did not show an increase in the mean green autofluorescence levels of each of the different cell populations analyzed, except for the monocytes ($P = 0.03$) (Fig. 4). In turn, lower fixative/sample volume ratios were associated with slight changes in the intensity of bcl2 staining for some populations of PB leucocytes: e.g. lymphocytes (but not the monocytes and the neutrophils) showed an increasing bcl2 MFI ($P = 0.0001$) (Fig. 4).

Progressively lower FSC and SSC values were observed for the neutrophils ($P \leq 0.01$), when identical numbers of PB cells in increasingly larger volumes of PBS-diluted PB—decreasing cell/protein concentrations and progressively lower fixative to PBS-diluted sample volume ratios—were analyzed (Fig. 4). In addition, fixation of progressively higher PBS-diluted PB sample volumes (in lower fixative to PBS-diluted sample volume ratios) was associated with increasingly higher bcl2 MFI values for the lymphocytes ($P = 0.01$). By contrast, no major changes were observed for the other cell populations analyzed, either for the FSC/SSC or for the green autofluorescence of PB leucocytes.

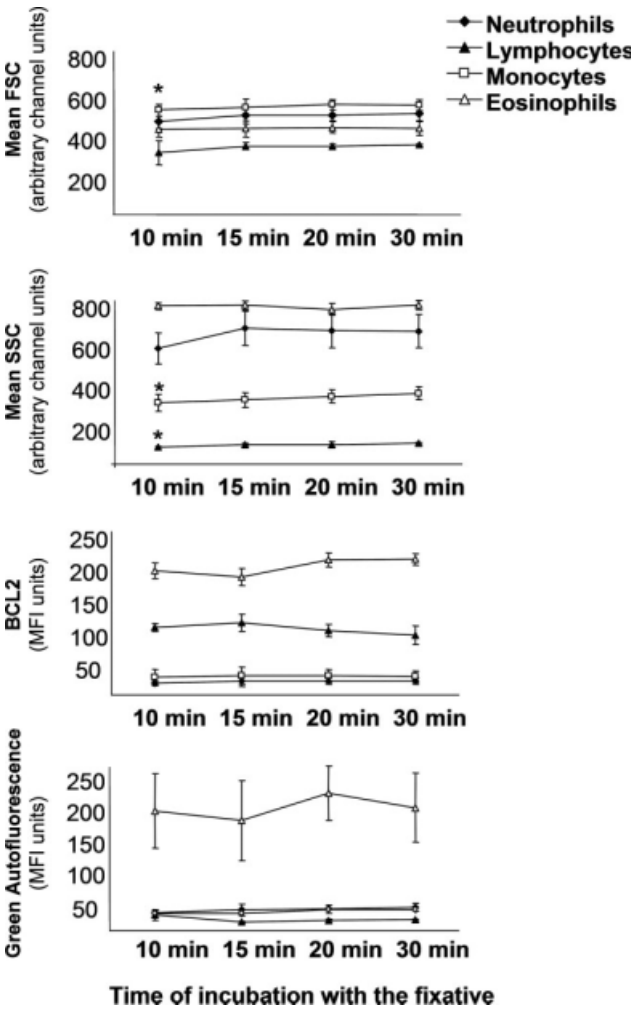

FIG. 3. Effect of the duration of the incubation with the fixative (10, 15, 20, and 30 min) on the light scatter characteristics (arbitrary linear mean channel values, scaled from 0 to 1023), the intensity of expression of bcl2 and the levels of FL1-associated green autofluorescence—arbitrary mean fluorescence intensity (MFI) values scaled from 0 to 10,000—of different populations of peripheral blood fixed and permeabilized leucocytes. Symbols represent the mean value and the vertical lines correspond to one standard deviation. Statistically significant differences were observed for the following comparisons: FSC ($P < 0.02$) and SSC ($P < 0.03$) of monocytes incubated with the fixative for 10 min versus all other time periods and; SSC of lymphocytes incubated for 10 versus 15, 20, and 30 min ($P < 0.03$).

Automated Correction of Light Scatter and Fluorescence-Associated Parameters

Automated correction of the position of individual events from a datafile derived from the measurement of

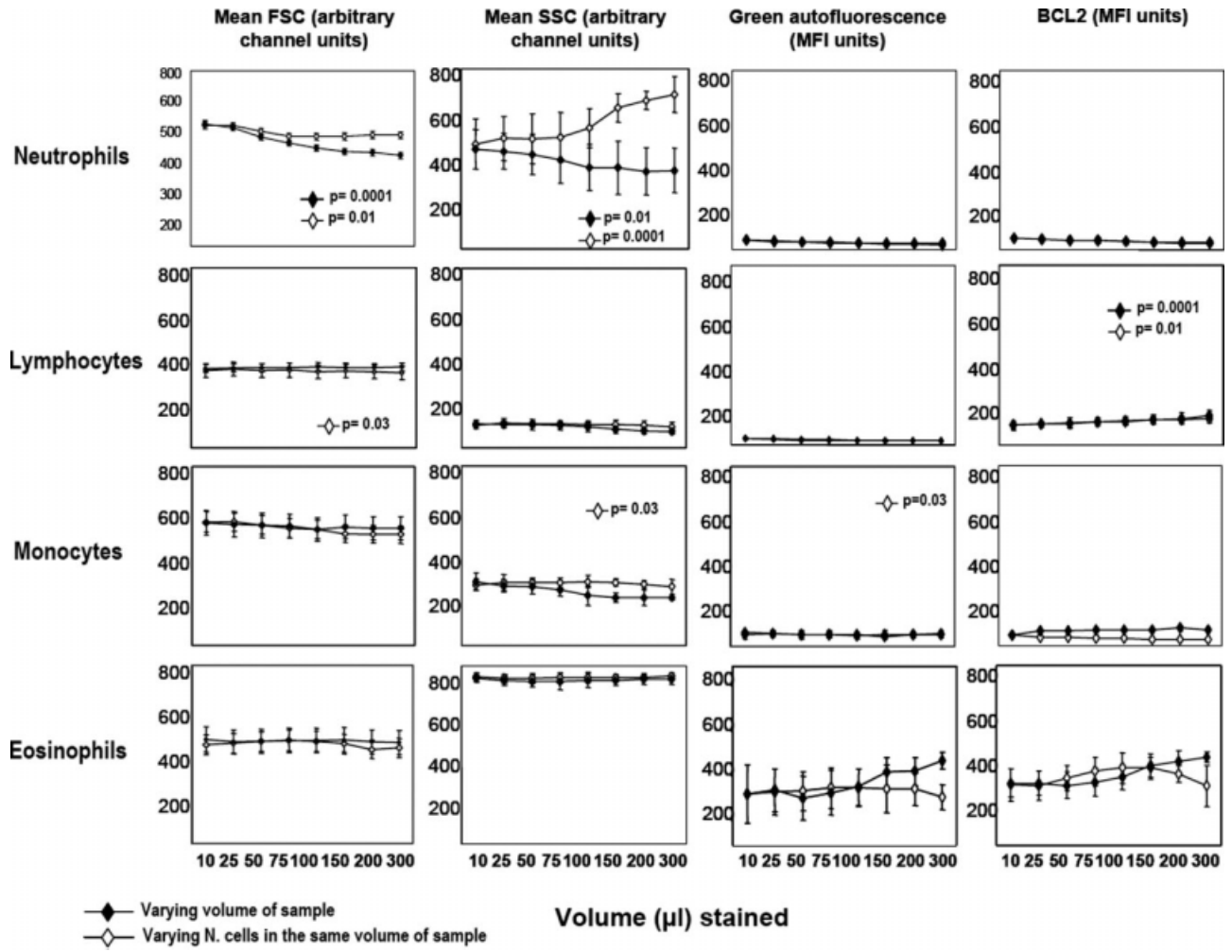

FIG. 4. Effect of both cell/protein concentration (—◆— symbols) and the fixative/sample (vol/vol) ratio (—◇— symbols) on the light scatter characteristics (arbitrary linear mean channel values, scaled from 0 to 1023), the intensity of expression of bcl2 and the levels of FL1-associated green autofluorescence—arbitrary mean fluorescence intensity (MFI) values scaled from 0 to 10,000—of different populations of peripheral blood fixed and permeabilized leucocytes. Symbols represent mean values and vertical lines correspond to one standard deviation of the mean. *P* values correspond to statistically significant differences observed after applying a paired test (Friedman test) for comparisons within each of the conditions (varying volume of sample and varying number of cells in the sample), identified by their corresponding symbols (—◆— and —◇—, respectively).

intracellular stainings, according to the position of the events corresponding to that cell population in a second datafile corresponding to a cell-surface staining of another aliquot of the same sample, is illustrated in Figure 5. As may be seen in it, the mean FSC and SSC values obtained for those sample aliquots that underwent intracellular staining procedures, were significantly different from the values corresponding to those events from the same cell populations treated with cell-surface-only staining techniques (Table 1). However, once the proposed correction strategy was applied, identical FSC/SSC and fluorescence distributions were seen for the same cell populations treated with cell-surface-only versus intracellular staining procedures (Table 1).

DISCUSSION

For many years, it is well-known that fixation/permeabilization is an essential step in allowing immunophenotypical analysis of intracellular antigens and proteins in individual cells (6,21–27). At present, several different fixation/permeabilization procedures are available for the flow cytometric characterization of intracellular proteins. However, the use of such fixation/permeabilization procedures is associated with some practical limitations (6,8–10,24–26,28–32). These limitations mainly relate to the fact that fixation of normal and leukemic cells is typically associated with significant changes in the light scatter properties and baseline autofluorescence levels of the cells of interest (6,8–10,25–30); in addition, with such techniques, binding of monoclonal antibodies to their cellular epitopes and/or the fluorescence emissions of fluorochromes already bound to the cell, are also negatively affected by the fixation procedure (8,9,26–28). Among other approaches, the use of the paraformaldehyde-based Fix & Perm™ reagent (24) (and the procedure recommended for its use)

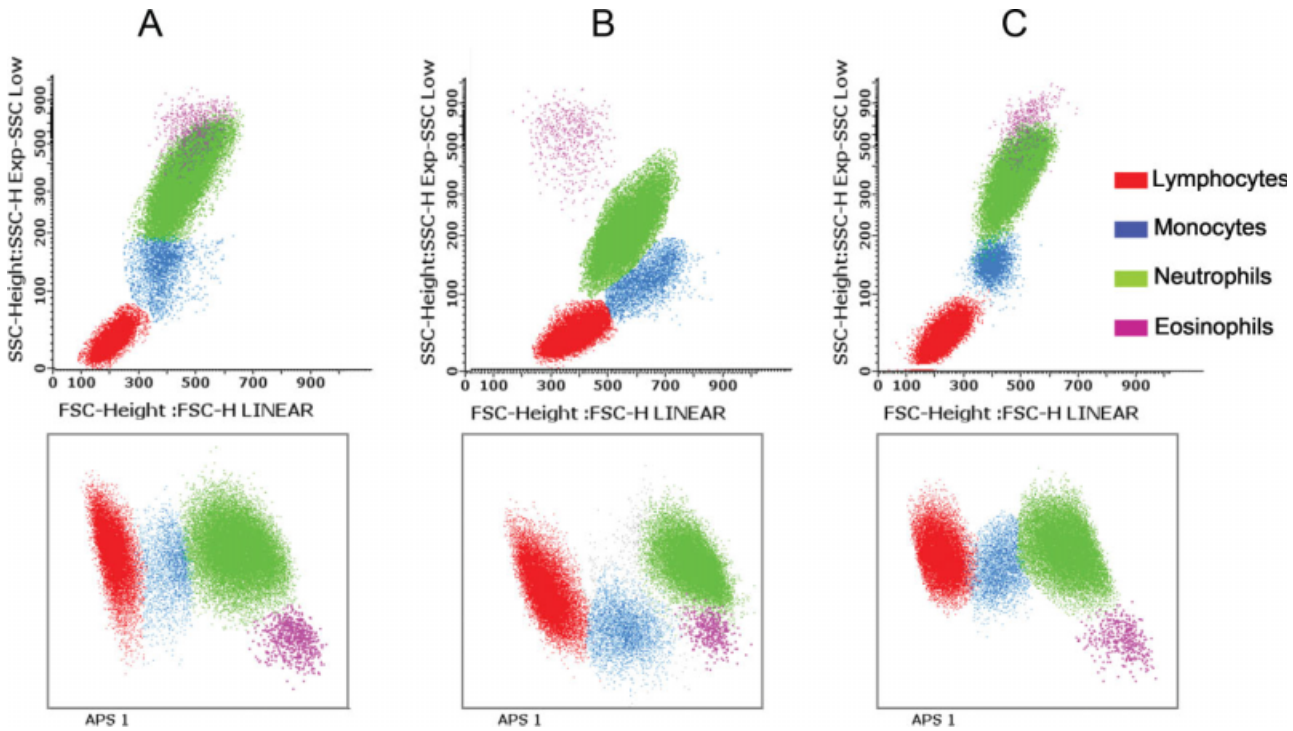

FIG. 5. Mathematical harmonization of common light scatter and green fluorescence features of cellular events corresponding to peripheral blood leucocytes treated with the Fix & Perm reagent according to the profiles observed for the same parameters in another aliquot of the same sample treated with a cell-surface staining-only protocol. Column A illustrates the light scatter (upper plot) and light scatter plus fluorescence characteristics—"automated population separator" view (APS view included in the INFINICYT software program) where individual events are represented based on vectors corresponding to a first versus second principal component analysis of the FSC, SSC, and green fluorescence values obtained for each individual cellular event in the corresponding data file (20)—(lower panel) of the different major populations of leucocytes present in an aliquot of a peripheral blood sample treated with cell surface-only staining procedures, whereas in Column B, the same flow cytometry parameters and profiles are shown for another aliquot of the same peripheral blood sample treated with intracellular staining procedures. In Column C, the same flow cytometry profiles are shown for the same cell populations and events displayed in Column B, after the automated correction proposed in this study was applied. For all columns, events corresponding to lymphocytes are represented in red, monocytes in blue, neutrophils in green, and eosinophils in violet. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

has become a gold standard for the intracytoplasmic staining of proteins expressed by hematopoietic cells and the evaluation of new intracellular staining reagents and techniques (6,8–10,25–30,33–35), for the diagnostic classification of leukemic cells in a hemato-oncology laboratory (5,6,8,10,28). In parallel, the use of the Fix & Perm™ reagent has turned into routine practice in many clinical diagnostic laboratories (33,34). Despite significant improvement brought by the Fix & Perm™ reagent into the staining of cytoplasmic proteins in hematopoietic cells, this and other similar reagents still show some suboptimal features. Accordingly, these reagents show a significant impact on the light scatter properties of nucleated PB cells, particularly the neutrophils' SSC, which may vary from one sample to another (6,9,10). Such variations represent a major limitation for the use of automated and semi-automated procedures for the analysis of multiparameter FCM data derived from panels of monoclonal antibodies which simultaneously include intracellular stainings and cell-surface-only combinations of monoclonal antibodies for the characterization of normal and leukemic hematopoietic cells (13).

In this study, we have explored the impact of several technical factors potentially involved in the occurrence of significant and variable changes in the light scatter and fluorescence characteristics of PB leucocytes, which particularly affect the neutrophils, and which are induced by cell fixation with the Fix & Perm™ reagent and protocol. Cytoplasmic staining for the bcl2 protein, present at variable levels in the different populations of PB leucocytes, was used as a model for the staining of an intracellular protein. Our major goal was not to compare different procedures and reagents used for the intracellular staining of multiple antigens, but to evaluate a new mathematical approach to automatically correct for the differences observed in the light scatter and fluorescence patterns of paired aliquots of the same sample treated with cell-surface-only versus intracellular staining procedures, based on the use of the Fix & Perm™ reagent.

Overall, our results clearly show that neither the anticoagulant used (heparin vs. EDTA) nor storage of samples for <24 h had a significant impact on the FSC, SSC, autofluorescence, and bcl2-FITC staining characteristics of fresh PB leucocytes; similarly, the duration of the

Table 1
Automated Harmonization of Flow Cytometry Light Scatter and Green Fluorescence Parameter Measurements for Intracellular Versus Cell-Surface Staining Procedures of Paired Aliquots of the Same Peripheral Blood Samples (n =5)

		Cell-surface staining (CSS)	Intracellular staining			
			Initial (InIS)		Corrected (CoIS)	
Cell subsets	Parameters	Mean	Mean	InIS versus CSS (*P*-values*)	Mean	CoIS versus CSS (*P*-values*)
Lymphocytes	FSC	222 ± 54	333 ± 18	0.08	222 ± 54	0.31
	SSC	29 ± 5	31 ± 6	0.14	28 ± 5	0.32
	Green autofluorescence	5 ± 5	8 ± 2	0.08	5.2 ± 3	0.89
Monocytes	FSC	404 ± 59	547 ± 48	**0.04**	404 ± 59	0.32
	SSC	119 ± 5	134 ± 14	**0.04**	119 ± 5	0.32
	Green autofluorescence	10 ± 2	16 ± 2	**0.04**	9 ± 2	0.46
Neutrophils	FSC	440 ± 22	500 ± 12	**0.04**	440 ± 22	1
	SSC	307 ± 22	454 ± 15	**0.04**	307 ± 23	1
	Green autofluorescence	9 ± 2	15 ± 1	**0.03**	9 ± 1	0.43
Eosinophils	FSC	447 ± 18	402 ± 60	0.23	447.1 ± 18	0.32
	SSC	487 ± 27	523.2 ± 51	0.22	487 ± 27	0.32
	Green autofluorescence	70 ± 18	77 ± 14	0.22	71 ± 18	0.18

Results are expressed as mean ± one standard deviation. For SSC, linear values (arbitrary channel units) were used for all calculations.
**P*-values associated with statistical significant differences ($\leq$0.05) for paired comparisons are highlighted in bold.

fixation period of PB samples also showed no significant impact on either the scatter or the fluorescence properties of the major populations of PB leucocytes analyzed, whenever incubation periods of $\geq$15 min were used. In contrast, changes in cell/protein concentration and/or fixative/sample (vol/vol) ratios had a major impact on the light scatter characteristics of most cell populations analyzed. In this regard, lower cell/protein concentrations and lower fixative to PBS-diluted sample (vol/vol) ratios were associated with progressively lower scatter values; such changes mainly involved the SSC properties of neutrophils. Based on our results, such changes could be attributed to a lower concentration of cells and/or proteins in the sample, because the use of increasingly higher fixative/sample volume ratios corrected for the changes observed when PB instead of PBS-diluted PB samples were used at the same fixative/sample versus fixative/PBS-diluted sample volume ratios. Despite this, both the cell/protein concentration and the fixative/sample (vol/vol) ratio had a minor impact on baseline green autofluorescence levels, with a null-to-mild increase in the bcl2-associated green fluorescence of some, but not all, major populations of PB leucocytes. Although definitive conclusions can not be obtained with staining for a single protein (e.g., bcl2), these results could suggest than even for the highest sample/fixative (vol/vol) ratios used, concentration of the fixative is still at optimal levels with mild effects on the staining profiles of the bcl2 protein (26,36–38).

Several reports have previously compared the performance of different cell fixation and permeabilization reagents for the staining of intracellular markers in distinct populations of normal and neoplastic hematopoietic cells (6–10,25,28–30,34,35); however, to our knowledge, this is the first report in which the impact of different variables associated with the sample and the fixation procedure are analyzed in detail for the Fix & Perm™ reagent kit. Overall, our results indicate that to avoid a major decrease in SSC values obtained for normal PB neutrophils, attention should be paid to the use of relatively high cell/protein concentrations and low fixative/sample (vol/vol) ratios. In our study, fixative/sample (vol/vol) ratios of between 1/1 and 1/2 provided the most reproducible staining results for the Fix & Perm™ reagent; further studies are required to confirm that staining for proteins other than bcl2 also remains at expected values under such experimental conditions.

However, even under such conditions, fixation/permeabilization-induced changes occur, which vary among different populations of PB leucocytes (e.g., monocytes vs. neutrophils). This, points out the need for alternative strategies to make results fully comparable for different aliquots of a sample treated with intracellular versus cell-surface-only staining procedures. In line with this, here, we propose and evaluate a new mathematical approach to correct for such scatter/fluorescence changes associated with the use of different sample preparation protocols for the multiparameter flow cytometric characterization of groups of individual events within a cell population. This new mathematical strategy used to make populations of events present in distinct aliquots of the same sample which were prepared differently (e.g., with cell-surface-only vs. intracellular staining procedures) comparable, uses one data file as the reference data file and mathematically plots individual events from specific cell populations in a second data file, to the multidimensional spaces occupied by the same cell populations in the former, reference data file. As shown, with this new strategy a high degree of similarity is obtained for the staining profiles of reference versus corrected data files for the different major populations of leucocytes coexisting in a PB sample. Thus, the proposed algorithm facilitates direct comparison among multiparameter FCM profiles of cell populations

contained in different data files corresponding to distinct aliquots of the same sample, and it could potentially be used for harmonization of data in all applications of multiparameter FCM for which more than one sample preparation technique is required. A requisite of the mathematical model proposed is that the corresponding cell populations—in the reference as well as in the other data files—to be harmonized have to be clearly and unequivocally identified using automated, semi-automated, or expert-based manual gating strategies.

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